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Oncogenic Ras blocks transforming growth factor- β –induced cell-cycle arrest by degradation of p27 through a MEK/Erk/SKP2-dependent pathway

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Objective. To examine whether oncogenic Ras affects transforming growth factor (TGF)- β –mediated cell-cycle arrest in hematopoietic cells and the downstream signal transduction pathway involved in the interference with TGF- β –induced cell-cycle arrest.

Materials and Methods. Two leukemic cell lines bearing N-Ras^{L61} mutations; HL-60 and TF-1, and the M1 cell line with wt Ras were investigated for their response to TGF- β . Signal transduction inhibitors, overexpression and RNA interference studies were performed to investigate the involvement of the various proteins.

Results. Although TGF- β signal transduction was not affected, G0-G1 arrest was absent in HL-60 and TF-1 cells due to the absence of p27. Overexpression of p27 restored TGF- β –induced cell-cycle arrest, as well as interfering in Ras-mediated signaling. The farnesyl transferase inhibitor L744832 and the MEK inhibitor U0126 both restored p27 levels and cell-cycle arrest in response to TGF- β . The absence of p27 protein is due to elevated levels of the ubiquitin ligase SKP2, which complexes with and targets p27 for degradation. RNA interference for SKP2 and treatment of these cells with the proteasome inhibitor MG132 restored p27 levels, corresponding with decreasing SKP2 levels after interfering in N-Ras signal transduction. P27, phosphorylated at threonine 187, is nuclear localized in N-Ras-containing cells. Mutation of this residue to alanine rendered p27 insensitive to degradation.

Conclusion. N-Ras^{L61} transformed cells lack a G0-G1 arrest upon TGF- β treatment due to absence of p27. p27 is degraded through a MapK-, and SKP2-dependent pathway. Overexpression of p27 results in restoration of cell-cycle arrest upon TGF- β treatment. © 2005 International Society for Experimental Hematology. Published by Elsevier Inc.

Transforming growth factor- β (TGF- β) and Ras-signal transduction have opposite effects on cell proliferation. Where Ras and downstream mitogen-activated protein kinase/extracellular signal regulated kinase kinase (MEK/Erk) signaling are induced in response to growth promoting factors [1,2], cell growth is arrested in response to TGF- β [3–5]. Binding of TGF- β to its receptor leads to activation of Smad proteins and modulates the transcription of many target genes, including genes that affect cell fate [3,4].

TGF- β –induced cell-cycle arrest is mediated by various cyclin-dependent kinase inhibitors (CDKi), including p15^{Ink4B} [6,7], p21^{Waf1/Cip1} [8,9], p27^{Kip1} [10,11], and p57^{Kip2} [12]. Furthermore, expression and activity of additional proteins, such as c-Myc, cyclin A, CDK4/6, and the phosphatase cdc25A, are suppressed by TGF- β [13,14]. These events eventually lead to decreased activity of the Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes and ultimately result in cell-cycle arrest.

One of the key intermediates in TGF- β –induced cell-cycle arrest is the CDKi p27^{Kip1} and alterations in protein levels or localization interfere with the inhibitory activity on CDK2 and TGF- β –induced cell-cycle arrest [13,15–17]. Regulation of p27 is primarily controlled by phosphorylation and subsequent degradation or proteolysis by two distinct

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pathways. Phosphorylation of p27 on Threonine residue 187 by the CDK2-Cyclin E complex targets p27 for ubiquitination in the nucleus by the E3 ubiquitin ligase SKP2 [18–24]. This ubiquitinated p27 is then degraded by the 26S proteasome [22,23,25]. Phosphorylation of p27 on other residues, such as Serine 10, Threonine 157, and Threonine 198, leads to cytoplasmic translocation, where p27 is either degraded or stabilized [26–32]. Cytoplasmic p27 has no inhibitory effect on the cell cycle.

Besides inactivating mutations in the TGF- β receptors or Smad proteins [3], TGF- β -mediated cell-cycle arrest can be blocked by oncogenic Ras. This has been attributed to MAP kinase-dependent phosphorylation of Smad 2/3 and subsequent impaired nuclear translocation, degradation of Smad 4, or mislocalization of p27 to the cytoplasm [13,33,34].

In acute myeloid leukemia (AML), cytoplasmic mislocalization of p27 is associated with constitutive Akt phosphorylation and unfavorable prognosis [35]. In addition, Akt phosphorylation might depend upon Ras activation [36]. Based on these data it is conceivable that oncogenic Ras, which is frequently mutated in AML, renders hematopoietic cells insensitive to TGF- β treatment through modulation of p27 function, resulting in a disturbed negative feedback control.

In this report, we demonstrate that the leukemic cell lines TF-1 and HL-60, bearing oncogenic N-Ras^{L61} mutations, are insensitive to TGF- β with regard to cell-cycle arrest. Surprisingly, instead of p27 mislocalization to the cytoplasm, we found that these leukemic cell lines were devoid of p27. A Ras/MEK/Erk/SKP2-dependent mechanism is responsible for degradation of p27 and overexpression of p27 in these cells restored TGF- β -induced cell-cycle arrest.

Materials and methods

Cell culture, (viral) vectors, and transfections

The human and mouse myeloblastic cell lines HL-60 (product no. CCL-240; ATCC, Manassas, VA, USA) and M1 (Product No. TIB-192, ATCC) were cultured in RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin (ICN Biomedicals, Costa Mesa, CA, USA) and 10% fetal bovine serum (FBS; Bodinco, Alkmaar, the Netherlands). The human erythroleukemia cell line TF-1 (product no. CRL-2003; ATCC) was cultured in RPMI 1640, supplemented with 10% FBS and 10 ng/mL granulocyte monocyte-colony stimulating factor (Genetics Institute, Cambridge, MA, USA).

pBabe FLAG-27 was constructed by ligating the EcoRI-XhoI fragment of pCDNA3 FLAG-p27 into the pBabe-Ires-GFP puro plasmid. TF-1 cells were transduced with viral particles collected from Phoenix cultures transfected with pCL-AMPHO and pBabe Flag-p27 using FuGENE6 (Roche, Almere, The Netherlands). Cells stable expressing FLAG-p27 were continuously selected with 1 μ g/mL puromycin (Sigma, St. Louis, MO, USA).

TF-1 cells were electroporated (960 μ F, 240 V) with 15 μ g of pCMV Tag2B or pCDNA3 FLAG wt p27 or S10A, T157A, S161A, T162A, S178A, or T187A mutant p27 in order to study degradation.

HL-60 and TF-1 cells were either mock electroporated (960 μ F, 240 V) or 1) with 12.5 μ g pGAL4-Elk1, 12.5 μ g pGAL4-TK-luc and 5 μ g pDM2-LacZ for the Elk-Gal4 transactivation assay, 2) with 12.5 μ g 7X Smad binding element (SBE) luciferase and 12.5 μ g pDM2-LacZ for the TGF- β reporter assay, or 3) with the p27GL-1609 reporter plasmid [37] in order to study p27 transcription.

All reporter transfections were collected in 100 μ L reporter lysis buffer (Promega Corp, Leiden, the Netherlands) and subjected to luciferase and β -galactosidase assays as described previously [38].

Reagents and antibodies

The p16, p27(C-19), p57(C20), P-p27 Ser10, Erk (K-23), N-Ras(C20), Rb(C15), and Smad4, antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Smad3, SKP2, p27, and P-p27 Thr187 were purchased from Zymed Laboratories (South San Francisco, CA, USA). P-Erk antibody was obtained from New England Biolabs (Beverly, MA, USA). Flag antibody, p21 antibody, and an antibody against Actin (C4) were obtained from Sigma, Transduction Laboratories (Lexington, KY, USA) and ICN Biomedicals, respectively. All antibodies were reactive for both human and mouse isoforms. Horseradish peroxidase-labeled secondary antibodies were obtained from DAKO (Glostrup, Denmark). The farnesyl transferase inhibitor L744832 and the MAP kinase/ERK kinase (MEK) 1 inhibitor U0126 were obtained from Biomol (Plymouth Meeting, PA, USA) and Promega Corp., respectively. TGF- β was purchased from R&D systems (Minneapolis, MN, USA). MG132 was obtained from Calbiochem (Darmstadt, Germany). Nocodazole, Actinomycin D, Cycloheximide, Puromycin, RNase A, propidium iodide (PI), and Triton X-100 were obtained from Sigma. Sodium citrate-dihydrate was purchased from Merck (Darmstadt, Germany).

Ras binding reaction

TF-1, HL-60, and M1 cells (5×10^6) were incubated in 2 mL RPMI 1640 medium supplemented with 0.5% FBS for 24 hours. Cells were lysed in 400 μ L lysis buffer and a Ras binding reaction was performed as described previously [36].

Mutation analysis of N-Ras

Mutation analysis of N-Ras was performed by single-strand conformation polymorphism (SSCP). Genomic DNA was extracted from HL-60 and TF-1 cells and polymerase chain reaction (PCR) analysis was performed as described previously [36,39].

Preparation of protein extracts and Western blot analysis

The amount of p27, p21, p16, p57, Erk, SKP2, Ras, FLAG, Smad3, Smad4, and actin, and the degree of phosphorylated p27 and Erk were determined by Western blotting on whole cell extracts, cytoplasmic, or nuclear extracts. Total cell extracts were prepared by resuspending the cells in lysis buffer (20 mM Tris-HCl, pH 7.6; 100 mM NaCl; 10 mM ethylenediaminetetraacetic acid [EDTA]; 1% NP-40; 10% glycerol; 2 mM Na₃VO₄; 2 mM phenylmethylsulfonyl fluoride [PMSF]; 1 μ M pepstatin; and 1 mM dithiothreitol [DTT]) and kept for 15 minutes on ice.

For cytoplasmic and nuclear extracts, cells were lysed according to the “mini extracts” method [40]. All extracts were normalized for protein content prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proper fractionation and lack of leakage of nuclear proteins to the cytosol was determined with Western blotting for the nuclear protein retinoblastoma.

Immunoprecipitation

Cells (2×10^7) were cultured for 24 hours in RPMI 1640 medium supplemented with 10% FBS. Cells were washed with ice-cold PBS and subsequently lysed in 500 μ L lysis buffer (20 mM Tris-HCl, pH 7.6; 100 mM NaCl; 10 mM EDTA; 1% NP-40; 10% glycerol; 2 mM Na_3VO_4 ; 2 mM PMSF; 1 μ M pepstatin; and 1 mM DTT) for 10 minutes on ice. Cell lysates were clarified at 5000g for 10 minutes and after preclearing with 30 μ L Protein A Sepharose beads for 1 hour at 4°C, cell lysates were incubated with 5 μ L SKP2 or p27 antibody (Zymed) and rotated for 4 hours at 4°C. Protein A sepharose beads (30 μ L) were added to each sample and incubated for 16 hours at 4°C. The immune complex was washed three times with lysis buffer. The immune complexes were heated in sample buffer, separated by SDS-PAGE, immunoblotted on polyvinylidene difluoride membrane (Millipore), and incubated for 16 hours with either anti-SKP2 or anti-p27 antibody. Immunocomplexes were detected using enhanced chemiluminescence (ECL).

Fluorescein-activated cell sorting

TF-1, HL-60, and M1 cells were synchronized with 0.33 μ M Nocodazole. After 16 hours cells were washed three times and plated at $0.25 \times 10^6/1.5$ mL and treated with TGF- β (2 ng/mL), 0.001% dimethylsulfoxide (DMSO), U0126 (10 μ M), or L744832 (50 μ M) as indicated. After 72 hours, cells were washed and incubated with PI solution (0.1 μ g/ μ L RNase A, 0.96 mg/mL sodium citrate dihydrate, 0.02 μ g/ μ L PI, 0.1% Triton X-100) for 20 minutes at room temperature. Binding of PI was measured using fluorescein-activated cell sorting (FACS; Becton Dickinson, Sunnyvale, CA) and cell-cycle analysis was performed using ModFit LT.

Quantitative PCR

RNA extraction, preparation of cDNA, and quantitative PCR (Q-PCR) for Smad7, SnoA, SKP2, p15, p18, and p27 were performed as described previously [41]. Primers used: Smad7 For gcctcgacagctcaattcg; Smad7 Rev cgtccacggctgtcgcataa; SnoA For cattctcacagatcacctgac; SnoA Rev gtcttactctctcacaggatg; SKP2 For ccagcaagacttctgaac; SKP2 Rev ggaggcacagacaggaaa; p15 For cgcgaggagaacaaggcat; p15 Rev gctcccgaacgggtgact; p18 For gattgccaggagactgctac; p18 rev cgtgtgcttcaccaggaact; p27 For gcaac-cgacgattctctac; p27 Rev gtccattccatgaagtcagc; HPRT For tggcgtcgtgattagtgtatg; HPRT Rev gatgtaatccagcaggtcag. Primers for SKP2 and the CDKi were designed against conserved regions between the human and mouse genes. Primers for Smad7 and SnoA are human-specific.

RNA interference

Short interfering RNA duplexes for SKP2 were made using the Silencer short interfering RNA (siRNA) construction kit from Ambion according to the manufacturers protocol (Austin, TX). The SKP2 target sequences used: AAAGAGGAGCCCGACAGTGAG and AAGTTGCAGAATCTAAGCCTG. GAPDH control siRNA duplexes are provided within the kit. TF-1 cells were transfected with a final concentration of 50 nM or 100 nM of RNA interference (RNAi) duplexes using oligofectamine according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). Lysates were made on the indicated time points in lysis buffer and equal amounts of protein were subjected to Western blot analysis.

Statistical analysis

The Student's *t*-test for paired samples was used to determine statistical significance of the cell-cycle data.

Results

p27 expression and TGF- β -induced

cell-cycle arrest in cells containing oncogenic Ras

The human myeloid leukemic cell line HL-60 and the erythroleukemic cell line TF-1, which have been reported to contain oncogenic N-Ras mutations [36,42], were studied with respect to TGF- β -mediated cell-cycle arrest. As a negative control, the myeloid cell-line M1 was used, because this cell line contains wt Ras. SSCP analysis confirmed that HL-60 and TF-1 cells are heterozygous for the N-Ras^{L61} mutation (data not shown). To determine whether Ras is activated in these cells, we performed Ras pull-down assays and determined activation of the downstream effector Erk [2] (Fig. 1a). In HL-60 and TF-1 cells activated Ras and phospho-p42/44 (P-Erk) were observed, both of which were absent in M1 cells, although antibodies were capable of recognizing mouse isoforms (Ras input lane and Erk, data not shown). To test the effect of TGF- β on the cell-cycle, cells were synchronized by nocodazole treatment for 16 hours and subsequently cultured in the absence or presence of TGF- β . FACS analysis demonstrated a minor or no increase in the percentage of cells in G0-G1 phase of the cell-cycle in response to TGF- β (HL-60: 46.3 ± 0.7 vs 53.1 ± 0.4 ; TF-1: 42.9 ± 1.2 vs 42.1 ± 0.4) (Fig. 1b). This in contrast to the M1 cell line, which demonstrates an increase of cells in G0-G1 phase from $48.9\% \pm 0.5\%$ to $76.4\% \pm 2.2\%$ in response to TGF- β .

Next, Western blot analysis was performed in order to determine whether the absence of cell-cycle arrest in the oncogenic Ras-containing cells is related to p27 dysfunction

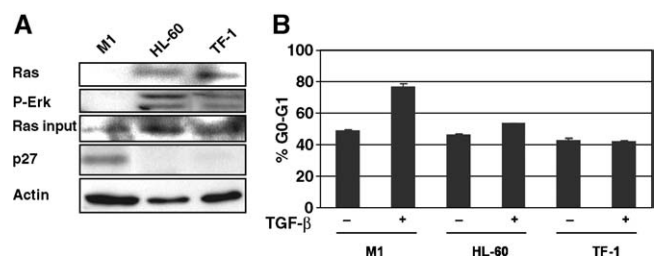


Figure 1. Cells containing N-Ras mutations are unresponsive to transforming growth factor (TGF)- β and are devoid of p27. (A) TF-1, HL-60, and M1 cell extracts were Western analyzed for activated Ras, P-Erk, and p27. After synchronization, cells were harvested and lysed in lysis buffer and a Ras binding reaction was performed as described in Material and Methods. Ras denotes activated Ras, which is precipitated with Raf-GDS glutathione-S-transferase-Ras binding domain precoupled to glutathione-sepharose beads. Total lysates show amounts of P-Erk, Ras, Actin, and p27 before precipitation. (B) M1, HL-60, and TF-1 cells were synchronized in M-Phase with Nocodazole and subsequently cultured with or without TGF- β as indicated. After 72 hours, cells were washed and incubated with PI solution. Binding of PI was measured using fluorescence-activated cell sorting and cell-cycle analysis was performed using ModFit LT. The percent of cells in G0-G1 phase is depicted as mean % G0-G1 \pm standard deviation.

[13]. In M1 cells, p27 was located in the nuclear compartment (data not shown and box Fig. 7b). In contrast, no p27 expression was observed in the N-Ras containing HL-60 and TF-1 cells (Fig. 1a). To exclude cell-cycle-dependent regulation of p27, presence of p27 was analyzed for all cell-cycle phases after synchronization. As expected, M1 cells show a cell-cycle-dependent regulation of p27, whereas p27 is absent in all cell-cycle phases in the HL-60 and TF-1 cell line (data not shown). These observations suggest that the absence of p27 in cells containing N-Ras mutations might be involved in the unresponsiveness toward TGF- β .

TGF- β signal transduction is not impaired in cells with oncogenic Ras

First, we determined whether HL-60 and TF-1 cells are able to respond to TGF- β or whether the decreased cell-cycle arrest is due to the absence of p27. Although oncogenic Ras has been described to be involved in degradation of Smad 4 [33], Figure 2a illustrates that Smad 4 protein is present in the HL-60 and TF-1 cell lines in levels comparable to M1 cells.

In addition, TGF- β signal transduction can be inhibited by modulating the cytoplasmic to nuclear translocation of Smad 2/3 through an Erk 1/2-dependent mechanism [34], which has been described to be activated by Ras in hematopoietic cells [2]. In the N-Ras-containing cells, Smad 3 nuclear accumulation was observed in response to TGF- β treatment (Fig. 2b), indicating that Ras does not interfere with cytoplasmic to nuclear shuttling of Smad 3.

Finally, TGF- β responsive target genes (including Smad 7 and SnoA) were studied by means of Q-PCR (Fig. 2C). In both HL-60 and TF-1 cells, TGF- β treatment resulted in an increased expression of these target genes. This observation was confirmed by a TGF- β reporter assay, where HL-60 and TF-1 cells were transfected with a reporter construct containing seven copies of a Smad responsive element (7X SBE-luciferase). Upon TGF- β treatment, transactivation of this reporter was observed in both cell lines (Fig. 2d). In conclusion, these data show that Smad-mediated TGF- β signaling is normal in these N-Ras containing cells.

Oncogenic Ras regulates p27 through a MEK/Erk-dependent pathway

To demonstrate a connection between Ras signal transduction and the absence of p27 expression, TF-1 and HL-60 cells were treated with inhibitors of the Ras (L744832) and MEK/Erk (U0126) pathways. As depicted in Figure 3a, a reappearance of p27 expression in TF-1 and HL-60 cells is observed in the presence of inhibitors U0126 and L744832. The effectivity of the inhibitors was confirmed by reduced phosphorylation of Erk 1/2 (Fig. 3a and densitometry Fig. 3b) and reduced downstream Elk signaling, as investigated by an Elk-Gal4 transactivation assay (Fig. 3b).

In order to demonstrate that the effect of Ras/MEK/Erk signaling is specific for the p27 protein, additional CDKs were studied. The CDKi p21^{Waf1} is absent in unstimulated hematopoietic cells [41], which was confirmed for the HL-60 cell line. Upon treatment with U0126, no change in

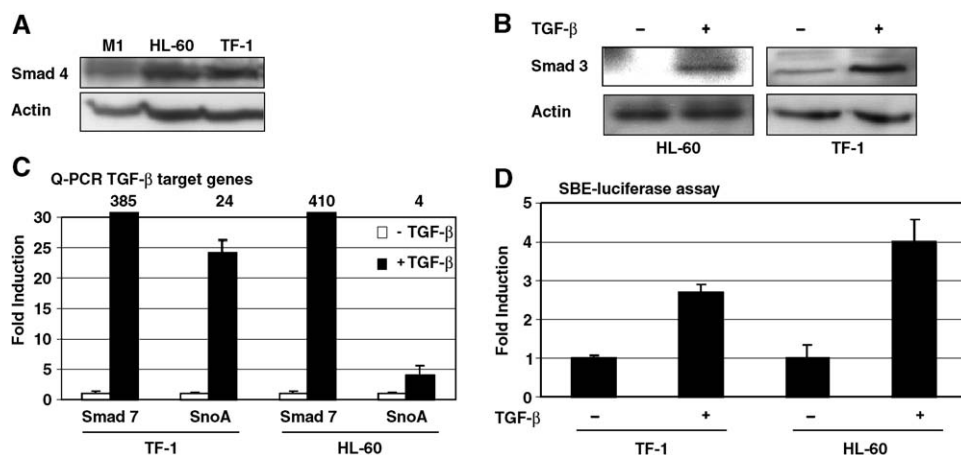


Figure 2. TGF- β signal transduction is normal in N-Ras containing HL-60 and TF-1 cells. (A) Smad 4 expression in M1, HL-60, and TF-1 cells. After 24 hours of culture, cell extracts were subjected to Western blot analysis for Smad 4 and Actin. (B) Smad 3 shows nuclear localization after 30 minutes of TGF- β treatment. HL-60 and TF-1 cells were treated with or without 2 ng/mL TGF- β . Nuclear fractions were isolated and subjected to Western blot analysis for Smad 3 and Actin. (C) TGF- β activates transcription of target genes in HL-60 and TF-1 cells. Cells were cultured with 2 ng/mL TGF- β for 1 hour and cDNA was prepared as described. Quantitative polymerase chain reaction for Smad 7 and SnoA was performed and normalized against expression of the common household gene HPRT. Fold inductions are presented as sample mean \pm standard deviation relative to untreated samples. (D) TGF- β transactivates transcription from a 7X SBE-luciferase construct. HL-60 and TF-1 cells were electroporated with a 7x SBE Luciferase vector and pDM2-LacZ. After 24 hours, cultures were split in two and treated for 16 hours with or without 2 ng/mL TGF- β . Cell extracts were subjected to luciferase and β -galactosidase assays as described. Luciferase values were normalized against β -galactosidase activity and fold inductions are presented as sample mean \pm standard deviation relative to untreated samples.

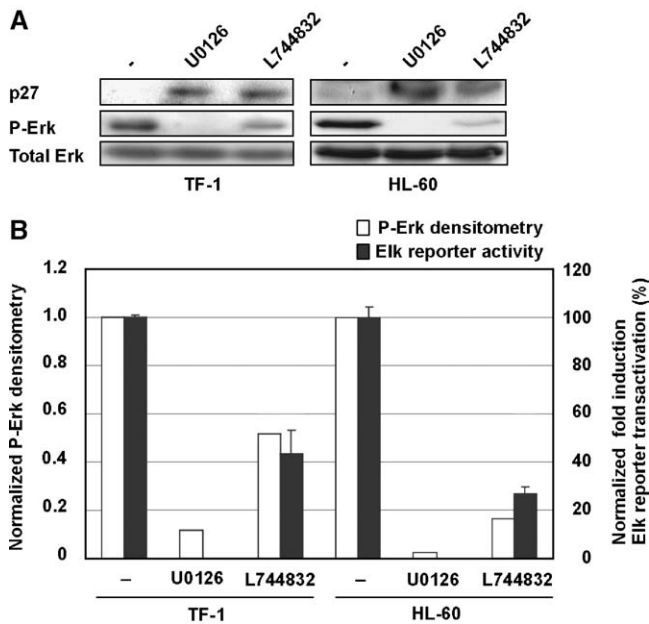


Figure 3. Inhibition of MapK and Ras signal transduction restores p27 levels in TF-1 and HL-60 cells. (A) Treatment with U0126 and L744832 restores p27 protein levels in TF-1 and HL-60 cells. Cells were cultured for 16 hours in the presence of 10 μ M U0126 or 50 μ M L744832. Cell extracts were subjected to Western blot analysis for P-Erk, total Erk, and p27. (B) Densitometry of P-Erk and Elk reporter activity indicate inhibition of Ras/MapK signaling by U0126 and L744832. White bars indicate P-Erk densitometry belonging to Fig. 3a. Black bars indicate normalized Elk reporter luciferase activity against β -galactosidase activity. HL-60 and TF-1 cells were either mock electroporated or with pGAL4-Elk1, pGAL4-TK-luc and pDM2-LacZ for the Elk-Gal4 transactivation assay. Fold inductions are presented as sample means \pm standard deviation relative to untreated samples.

expression of p21 protein was observed (Fig. 4). TF-1 cells express p21^{Waf1} (Fig. 4, first lane), which disappears in the presence of U0126, while no change was observed in the expression of the CDKi p57^{Kip2}, as is the same for HL-60 cells (Fig. 4). The expression of the CDKi p16^{INK4a} is absent in HL-60 cells, without changes upon U0126 treatment, whereas in TF-1 cells p16 protein is expressed, which appears to decrease slightly upon U0126 treatment (Fig. 4). Q-PCR analysis for the expression of the CDKis p15^{INK4b} and p18^{INK4c} demonstrated no significant changes upon U0126 treatment (data not shown).

Although some changes are observed for p21 and, to a lesser extent, p16 upon U0126 treatment, the most prominent effect was observed on p21 expression levels, which were strongly reduced by U0126 treatment and this would positively affect cell-cycle progression. These data strongly suggest that the blockade of TGF- β -induced cell-cycle arrest by Ras/MEK/Erk signaling in TF-1 and HL-60 cells is mediated through modulation of the p27 expression levels, because none of the other CDKis are upregulated after U0126 treatment.

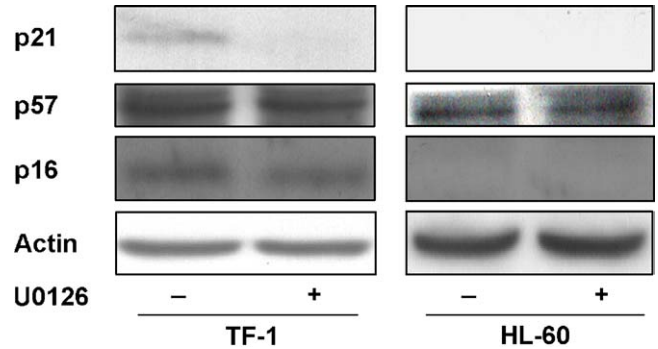


Figure 4. Inhibition of MapK signal transduction does not upregulate other cyclin-dependent kinase inhibitors. The CDKi p21 disappears upon U0126 treatment and the expression of the CDKi's p16 and p57 is not changed upon treatment with U0126. TF-1 and HL-60 cells were cultured for 16 hours in the presence or absence of 10 μ M U0126. Cell extracts were subjected to Western blot analysis for p21, p57, p16, and Actin.

TGF- β -mediated cell-cycle arrest is restored after U0126 and L744832 treatment and is p27-dependent

To demonstrate that reappearance of p27 is associated with TGF- β -mediated cell-cycle arrest, TF-1 cells were stimulated with TGF- β in the presence of U0126 or L744832. A significant increase in the percentage of cells in G0-G1 phase (Fig. 5a, 13.6% and 7.2% respectively, * and ** = $p < 0.05$) was demonstrated by FACS analysis.

To study the specificity of this effect, a TF-1 cell culture was generated in which the cells stably express p27 through retroviral transduction with pBabe-Flag-p27. In these cells, cell-cycle analyses were performed with TGF- β in the absence and presence of the MEK/Erk inhibitor U0126. Mock (pBabe) transfected TF-1 cells have a minor increase in the percentage of cells in G0-G1 phase after TGF- β treatment (Fig. 5b, 7.2%, $p < 0.05$) and this increase can be enhanced by inhibiting the MEK/Erk pathway (20.8 %, $\bullet = p < 0.05$). TF-1 cells expressing pBabe-Flag-p27 already show an almost twofold increase in the percentage of cells in G0-G1 phase (21.6%, $\bullet\bullet = p < 0.01$) in response to TGF- β , which was not further enhanced by U0126 treatment (18.3 %, $\bullet\bullet\bullet = p < 0.01$) (Fig. 5b). These results clearly indicate that the presence of p27 is sufficient to increase the percentage of cells in G0-G1 phase upon TGF- β treatment, although the percentages of untreated cells in G0-G1 phase varied between the different studied groups. Together, these data demonstrate that oncogenic Ras inhibits TGF- β -mediated growth arrest through modulation of p27 levels.

p27 is not regulated at the transcriptional level, but at the degradation level

p27 protein levels can be regulated at different levels, e.g., transcription, translation, or degradation [43]. To study how p27 protein levels are modulated, cells were pretreated with the RNA polymerase II inhibitor Actinomycin D. Although Actinomycin D was effective because it completely blocked TGF- β -induced transcription of Smad 7 (Fig. 6b),

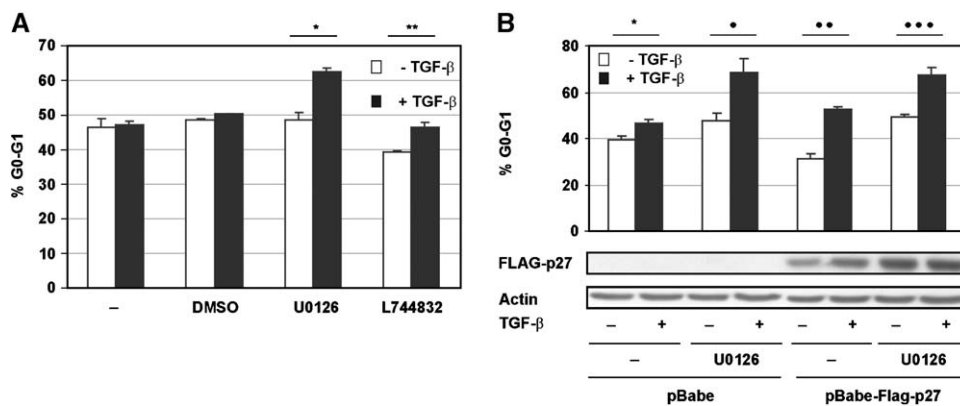


Figure 5. TGF- β -mediated cell-cycle arrest is p27-dependent and can be restored by inhibition of MapK and Ras signal transduction. (A) TGF- β -mediated cell-cycle arrest can be restored upon inhibition of the MapK and Ras signal transduction pathways. TF-1 cells were synchronized in M-Phase and subsequently cultured with or without TGF- β in the presence of dimethylsulfoxide, U0126, or L744832 as described. After 72 hours, cells were washed and incubated with PI solution. Binding of PI was measured using fluorescence-activated cell sorting and cell-cycle analysis was performed using ModFit LT. The percent of cells in G0-G1 phase is depicted as mean % G0-G1 \pm standard deviation. * and ** = $p < 0.05$. (B) Stable overexpression of FLAG-p27 is sufficient to restore TGF- β -mediated cell-cycle arrest. TF-1 cells were either mock (pBabe) stable transfected or with pBabe-FLAG-p27 and selected for positive cells with puromycin. The cells were synchronized in M-Phase and subsequently cultured with or without TGF- β or U0126. After 72 hours, cell-cycle analysis was performed as described above. The percent of cells in G0-G1 phase is depicted as mean % G0-G1 \pm standard deviation. * and • = $p < 0.05$, ** and *** = $p < 0.01$. Cell extracts were also subjected to Western blot analysis for FLAG and Actin.

p27 still reappeared after U0126 and L744832 treatment (Fig. 6a, middle panel, compare with left panel, the control for p27 reappearance). No significant upregulation of p27 transcription after U0126 treatment was observed with Q-PCR studies for p27 mRNA, which was confirmed by reporter assays with the p27GL-1609 construct [37] in the TF-1 and HL-60 cells (data not shown).

Treatment with the proteasome inhibitor MG132 induced reappearance of the p27 protein (Fig. 6a, right panel), suggesting that oncogenic Ras regulates p27 protein levels through degradation. In view of these data, the mRNA and protein expression levels of SKP2, the E3 ubiquitin ligase of p27, were analyzed. As depicted in Figure 6c and d, in the HL-60 and TF-1 cell lines SKP2 expression levels are fourfold increased as compared to M1 cells.

Subsequently, we determined whether Ras signaling enhanced SKP2 levels via an Erk-dependent pathway resulting in p27 degradation. Treatment of HL-60 cells with the Ras inhibitor L744832 resulted in reduced levels of phosphorylated Erk, downregulation of SKP2 expression and the reappearance of p27 protein (Fig. 6e). In addition, M1 cells transiently transfected with pCDNA3-Myc-N-Ras^{L61} demonstrated higher SKP2 protein levels than mock transfected control cells (data not shown). These data suggest that oncogenic Ras promotes p27 degradation through the activation of Erk and subsequent upregulation of SKP2 expression.

p27 Regulation involves degradation

Degradation of p27 has been described to involve two major steps, phosphorylation and ubiquitinylation [18]. Phosphorylation of p27 at Threonine 187 has been shown to occur through Cyclin E/CDK2 complexes in the nucleus, followed by binding of the F-box protein and E3 ubiquitin ligase

SKP2 [19–23]. Phosphorylation of p27 at Serine 10 also has been shown to occur by a yet unknown kinase, followed by nuclear export and degradation in the cytoplasm [30,31, 44–46]. In order to investigate the mechanism involved in p27 degradation in N-Ras-containing cells, alanine point mutants from known and putative phosphorylation sites, were transiently transfected into TF-1 cells. After 24 hours, cultures were split and treated with U0126 (Fig. 7a). Although to slightly different levels, treatment with U0126 resulted in reappearance of wild-type (Wt) and all p27 point mutants proteins. Strikingly, the p27^{T187A} mutant was resistant to degradation in TF-1 cells (Fig. 7a, upper and lower panel), indicating that this phosphorylation site is relevant for p27 degradation in the presence of oncogenic Ras.

Subsequently, degradation of p27 was studied in more detail. Virally transduced Flag-p27 TF-1 cells were treated with both the proteasome inhibitor MG132 and the translation inhibitor cycloheximide. This provides the opportunity to investigate steady-state levels of p27 in the cell. In addition, it is possible to study whether p27 is phosphorylated and whether degradation of p27 is a nuclear event.

After 18 hours of culture, p27, phosphorylated at serine 10 and threonine 187, was confined to the nuclear compartment without modulation by U0126 treatment (Fig. 7b). In addition, Flag-p27 was only detected in the nuclear compartment (Fig. 7b), suggesting that degradation of p27 occurs solely in the nucleus and no nuclear to cytoplasmic translocation takes place. The box next to panel B demonstrates the absence of p27 in both nuclear and cytoplasmic fractions in untreated parental TF-1 cells. M1 cells are shown as control. Moreover, these findings implicate that the phosphorylation of T187 is a prerequisite for p27 degradation, but it is

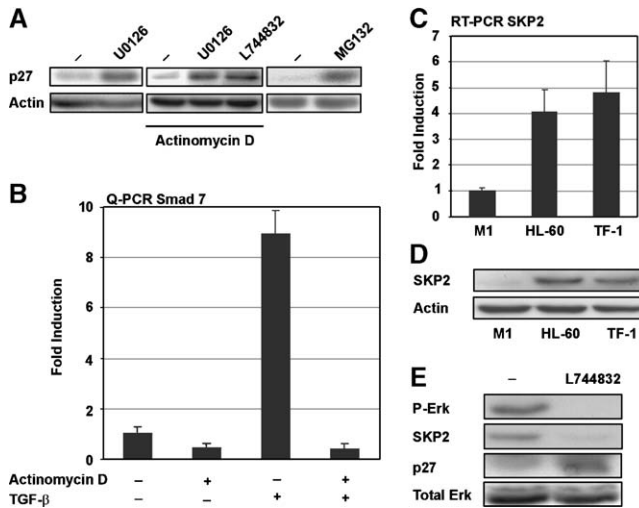


Figure 6. p27 is not regulated at the transcriptional level but at the degradational level in N-Ras containing cells and SKP2 is regulated by a Ras-dependent pathway. (A) Actinomycin D does not affect p27 restoration after U0126 and L744832 treatment and p27 levels are restored after treatment with the proteasome inhibitor MG132. TF-1 cells were cultured for 16 hours in the presence or absence of U0126, L744832, or 0.25 μ M MG132 with or without 0.50 μ g/mL Actinomycin D. Cell extracts were subjected to Western blot analysis for p27 and Actin. (B) Actinomycin D is effective in blocking TGF- β -induced transcription of Smad 7. TF-1 cells were cultured with 2 ng/mL TGF- β for 1 hour, with or without pretreatment with 0.50 μ g/mL Actinomycin D. cDNA was prepared as described and Q-PCR for Smad 7 was performed and normalized against expression of the common house hold gene HPRT. Fold inductions are presented as sample means \pm standard deviation relative to untreated samples. (C) Q-PCR for SKP2 indicates higher mRNA levels in N-Ras containing HL-60 and TF-1 cells. Cells were cultured and cDNA was prepared as described. Q-PCR for SKP2 was performed and normalized against expression of the common household gene HPRT. Fold inductions are presented as sample mean \pm standard deviation relative to M1 control cells. (D) Western blot analysis for SKP2 indicates higher protein levels in N-Ras containing HL-60 and TF-1 cells. Cells were cultured and cell extracts were subjected to Western blot analysis for SKP2 and Actin. (E) Inhibition of Ras signal transduction decreases P-Erk and SKP2 levels and increases p27 levels. HL-60 cells were cultured with or without L744832. After 16 hours, cells were lysed and cell extracts were subjected to Western blot analysis for P-Erk, total Erk, SKP2, and p27.

unlikely to be the cause of degradation. It is more conceivable that the elevated SKP2 levels are connected with the increased degradation of p27. Subsequently, coimmunoprecipitation studies were performed in the virally transduced Flag-p27 TF-1 cell line, to demonstrate complex formation between p27 and SKP2. Indeed, p27 can be precipitated with SKP2 (Fig. 7c, Left panel) and, vice versa, SKP2 precipitates with p27 (Right panel), indicating that these two proteins are in the same complex in the TF-1 cell line.

Finally, to study whether SKP2 is involved in p27 degradation in N-Ras-containing cells, RNAi studies for SKP2 were performed in the TF-1 cell line. Knockdown of SKP2 resulted in a reappearance of p27 protein, without an effect on GAPDH or actin levels (Fig. 7d). Conversely, siRNA oligos for GAPDH only affect GAPDH expression

levels and had no effect on p27. These data further support a role for SKP2 in p27 degradation.

Discussion

Loss of responsiveness to antiproliferative signals of growth factors, such as TGF- β , might provide a growth advantage to malignant cells [47]. Here we show that leukemic cells that carry an oncogenic N-Ras^{L61} mutation escape TGF- β -mediated cell-cycle arrest through nuclear degradation of the CDKI p27^{Kip1/Waf1} in a Ras/MEK/Erk/SKP2-dependent manner. This finding highlights an alternative pathway that results in TGF- β unresponsiveness. Among these alternative pathways are phosphorylation of Smad 3 and subsequent inhibition of nuclear translocation of this protein [34], the degradation of Smad 4 [33], and mislocalization of p27 to the cytoplasm [13].

In this study, we demonstrate that cells with N-Ras mutations have elevated SKP2 protein levels, a link that has been observed before [48]. In other cellular settings, SKP2-independent regulation of p27 has been reported, which coincided with Erk activation and involved translocation of p27 to the cytoplasm [49]. Phosphorylation on different residues has been demonstrated to be involved in cytoplasmic localization and/or stabilization of p27, e.g., Serine 10, Threonine 157, and Serine 178 [27,29,30,50]. In our experiments, we observed that alanine point mutants of S10 and S178 are upregulated to higher levels than other mutants by U0126 treatment (Fig. 7a), suggesting additional cytoplasmic degradation routes of p27. However, we show that p27 is not translocated to the cytoplasm, even though p27 is phosphorylated on residue S10 (Fig. 7b). The observed differences in expression between the various alanine point mutants are therefore likely to be the results of different transfection efficiencies. The only phosphorylation mutant that was resistant to degradation was the p27^{T187A} mutant, suggesting SKP2-dependent nuclear degradation [18–23, 30,31,46]. However, inhibition of the MEK/Erk pathway did not modulate phosphorylation of the T187 residue, suggesting that p27 phosphorylation is a prerequisite for binding to SKP2, but not the primary cause for the degradation process. Upregulation of SKP2 expression and binding to p27 in the nucleus seems to be the primary cause of the increased degradation of p27. The observed difference between our results and additional studies [49] is therefore likely to be cell-type-dependent.

Recently, TGF- β was demonstrated to destabilize SKP2 by ubiquitin-mediated proteolysis and decreased levels of Cks1 mRNA [51]. This, in turn, stabilized the p27 protein and resulted in cell-cycle arrest. In our study, however, TGF- β treatment of Wt TF-1 cells did not lead to an increase in p27 levels, probably because oncogenic Ras upregulates SKP2 to such levels that reduction of SKP2 by TGF- β is not sufficient to prevent p27 degradation by SKP2. Although it is reported that deletion of p27 causes hyperproliferation

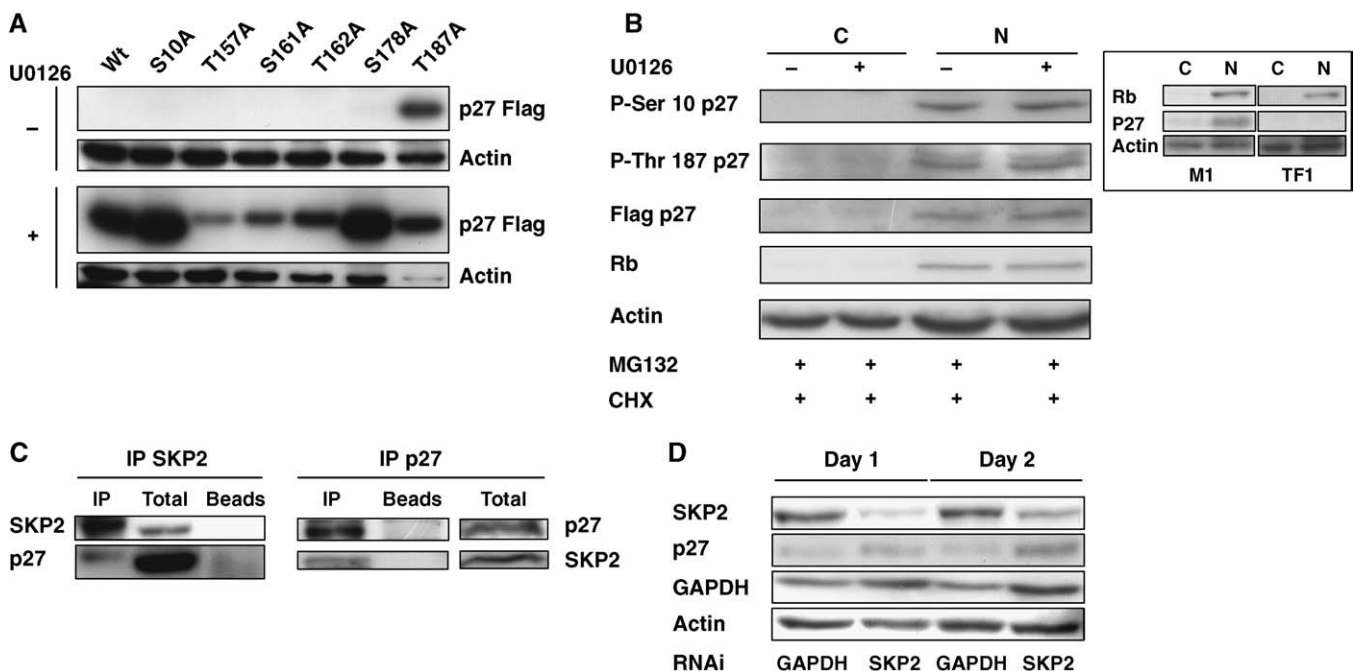


Figure 7. p27 protein is degraded in a Phospho Threonine 187/SKP2-dependent manner. (A) Mutant p27^{T187A} is resistant to degradation. TF-1 cells were electroporated with pCMV Tag2B or pCDNA3 FLAG wt p27 or S10A, T157A, S161A, T162A, S178A, or T187A mutant p27. After 24 hours, cultures were split in two and for 16 hours treated with or without U0126. Cell extracts were subjected to Western blot analysis for FLAG and Actin. (B) p27 is phosphorylated on S10 and T187 and is specific nuclear localized. Stable expressing pBabe-FLAG-p27 TF-1 cells were cultured for 16 hours in the presence of 0.25 μ M MG132 and 100 μ g/mL cycloheximide (CHX) with or without 10 μ M U0126. Cytoplasmic and nuclear fractions were isolated and subjected to Western blot analysis for Flag p27, phospho Ser10 p27, phospho Thr187 p27, Rb, and Actin. The box demonstrates the absence of p27 from both nuclear and cytoplasmic fractions in TF-1 cells. M1 cells are shown as a control. (C) SKP2 and p27 form a complex in N-Ras-containing TF-1 cells. Stable expressing pBabe-FLAG-p27 TF-1 cells were cultured for 16 hours and cell extracts were subjected to coimmunoprecipitation (IP) studies between SKP2 and p27 as described in Material and Methods. The precipitates were subjected to Western blot analysis for SKP2 or p27. IP indicates samples subjected to IP procedure, total indicates samples before IP procedure and beads indicates IP procedure without antibody to exclude aspecific binding. (D) p27 protein is reappearing after RNA interference for SKP2. TF-1 cells were transfected with siRNA oligos for SKP2 and GAPDH as described and at the indicated time points cell were lysed and extracts were subjected to Western blot analysis for SKP2, p27, GAPDH, and Actin.

[52], overexpression of FLAG-p27 itself is not enough to induce growth arrest in TF-1 cells. This suggests the activation of an additional signal transduction pathway by TGF- β . Alternatively, there is a persistent disbalance in the SKP2 and p27 levels, resulting in an inappropriate activation of cell-cycle progression. In the stable FLAG-p27 cells, an increased expression of p21 was observed and in some experiments p15 (data not shown). Because p27 has been demonstrated to enhance Sp1 stability and promoter binding [53] and both p21 and p15 expression is enhanced by Sp1 [54], upregulation of p21 and p15 could be an effect of p27-mediated Sp1 activation. These two CDKs might contribute to p27-mediated growth arrest in response to TGF- β , but they are downstream of p27.

P27 protein degradation is complex and controlled by various mechanisms. In this report, we show that p27 protein levels are regulated by degradation through a T187 phosphorylation-dependent, MEK/Erk-dependent, SKP2 pathway. Different signaling pathways downstream of MEK/Erk have been identified that affect SKP2 expression. Cul1, a critical component of the SCF(SKP2) ubiquitin ligase is

a direct transcriptional target of c-Myc [55]. It has been demonstrated that Myc expression and activity is downregulated upon MEK1 inhibition in conjunction with upregulation of p27 [56]. Indeed, inhibition of Ras activity with L744832 resulted in decreased c-Myc protein levels (data not shown), in accordance with reduced SKP2 levels and restoration of p27 levels (Fig. 6e). Alternatively, the SKP2 gene has been demonstrated to be a transcriptional target of GA binding protein (GABP) [57] and DNA microarray studies have indicated that GABP is a target gene of MEK/ERK signaling [58]. Both possibilities support our findings that 1) MEK/Erk signaling does not directly influence the phosphorylation status of p27 nor the transcription of p27 and 2) that delayed upregulation of p27 upon inhibition with U0126 and L744832 indicates an indirect effect of MEK/Erk signaling.

The inhibitory effect of p27 on the cell cycle is not restricted to the effects of TGF- β . Similar findings have been observed in solid tumors in response to vitamin D and retinoic acid [59,60]. Because N-Ras transformed teratocarcinoma and prostate epithelial cells are resistant to retinoic

acid [61,62] the previously described mechanism of disturbed p27 degradation widens the extent of enhancing the proliferative capacity of cells bearing oncogenic Ras mutations. Recently, studies demonstrated that unfavorable treatment results in AML were associated with elevated SKP2 levels and with a high cytoplasmic to nuclear p27 ratio [35,63]. Although in our model, cytoplasmic p27 was not observed, the high cytoplasmic to nuclear ratio in these studies might be due to nuclear degradation of p27 in a SKP2-dependent manner, thereby increasing the cytoplasmic p27 relative to the nuclear p27 levels. Ras-GTP and downstream targets can modulate these pathways in several ways, because Ras activation can be triggered directly by mutations in the *Ras* oncogene, or indirectly by mutations in the *PTPN11* gene, encoding for the protein tyrosine phosphatase SHP-2 [64] (and references therein), indicating the complexity of the mechanisms leading to oncogenic transformation and proliferation.

Recently, different phase I-II studies have been initiated to interfere with Ras-mediated signaling in AML patients and to increase the susceptibility of the leukemic cells for the cytotoxic effects of chemotherapy [65–67]. This report indicates that this is not the only advantage of this approach. AML cells might also become more susceptible for negative regulatory molecules that limit cell proliferation, including TGF- β .

In conclusion, we demonstrate that hematopoietic cells with N-Ras mutations do not respond to the inhibitory effects of TGF- β on cell-cycle progression, as a result of the absence of p27. This is due to a constitutive nuclear degradation of p27 in a Ras/MEK/Erk/SKP2-dependent manner.

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